Transcription Factors Positively and Negatively Regulating the Na,K-ATPase α1 Subunit Gene

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A cDNA clone encoding a zinc finger protein (AREB6) was isolated from a HeLa cell expression library using a positive regulatory element (−102 to −58) of rat Na,K-ATPase α1 subunit gene (Atp1α1) as a probe. The clone is apparently an extended one of Nil-2-a originally isolated as a negative regulator of interleukin 2 gene [Williams, T.M. et al. (1991) Science 254, 1791-1794]. The open reading frame encodes 1,124 amino acids. It contains 7 zinc-finger motifs arranged in two widely separated clusters. A glutamic acid-rich region is observed at the C terminus from residues 989 to 1123. Co-transfection of the AREB6 cDNA with Atp1α1 fused to a reporter luciferase gene indicated that the AREB6 protein enhances or represses the promoter activity of the gene depending on the quantity of cDNA and on the cell type. The mRNA of AREB6 is expressed in heart and skeletal muscle, but not in liver, spleen, or pancreas. Genomic Southern analysis indicated that the gene encoding AREB6 is present as only one copy or two at most. Another cDNA clone obtained by using the same probe was identified as HEB [Hu, J.S., Olson, E.N., & Kingston, R.E. (1992) Mol. Cell. Biol. 12, 1031-1042]. Co-transfection of the cDNA enhanced or repressed the promoter activity of Atp1α1 depending on the cell type. The binding regions of AREB6 and HEB are distinct from those of C1, C2, and C3 that were identified as binding complexes to ARE by gel retardation analysis using MDCK and B103 cell nuclear extracts [Suzuki-Yagawa, Y., Kawakami, K., & Nagano, K. (1992) Mol. Cell. Biol. 12, 4046-4055].

Na,K-ATPase is the enzyme responsible for maintaining the Na+ and K+ gradient across the cell membrane using the energy of ATP hydrolysis. The enzyme is composed of two subunits named α, the catalytic subunit and β, whose function is unclear. Three isoforms of each subunit have been identified so far (1-4). The α1 subunit is produced in all cell types so far examined and is thought to play an essential role in the maintenance of cell homeostasis. The α1 subunit gene was cloned and sequenced from horse (5), human (6), and rat (7). Analysis of regulatory elements of the rat gene revealed that ARE (−102 to −61 of the Atp1α1; transcription initiation site as +1) acts as a positive regulatory element in five different cell lines of various tissue origins. Gel retardation analysis indicated that two or three specific gel retardation complexes are formed, using various cell nuclear extracts (8). Two of the complexes (C1 and C2) were common to all cell types so far examined, while C3 was specific to MDCK, HeLa, and L6. For the purpose of identifying the binding proteins to ARE, we performed Southwestern screening of a HeLa cell expression library, using a DNA fragment containing the ARE sequence as a probe. Binding regions and G-residue contacts of the proteins coded by the isolated cDNA clones were analyzed by DNase I footprinting and methylation interference analyses. The effect on the Atp1α1 promoter activity was analyzed by co-transfection assay.

MATERIALS AND METHODS

Materials—Biochemical reagents were from Nacalai Tesque, Wako Chemicals, and Boehringer. Molecular biological reagents were from Toyobo and Takara. Radioisotopes were from Amersham. Cell culture plasticware was purchased from Corning, and media from Gibco and Cell Culture Laboratories.

Isolation and Sequencing of cDNA Clone—A total of 8 × 10⁶ plaques of a HeLa cell expression library (made from HeLa cell mRNA using the BRL Superscript Lambda System) were plated on nylon membranes (Hybond-N, Amersham) and screened with a PvuII-MluI (−102 to −58) fragment of rat Atp1α1 labeled with Klenow fragment. The probe was incubated with filters at 4°C for 2 h in the binding buffer (20 mM HEPES pH 7.9, 60 mM KCl, 4 mM MgCl₂, 5% glycerol, and 1 mM DTT) containing 40 μg/ml herring testis DNA and 0.25% non-fat dried milk (NFDM). After binding, filters were washed 3 times with the binding buffer containing 0.25% NFDM at 4°C (9). For obtaining the 5′-extended clone of AREB6s, the primer 5′-CATGCTGGTGTACCCCA (corresponding to the 5′ region of AREB6a) was synthesized and a primer extension library was constructed with use of the λgt10 cloning system (Amersham). Then 7.5 × 10⁴ phages were screened with SaII (in the phage vector)-HinfI fragment (containing
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Fig. 1. (A) Nucleotide sequence and the deduced amino acid sequence of AREB6. The 4th nucleotide is numbered +1. Positions of nucleotides are shown above each line and those of the deduced amino acids are indicated on the left side. Putative zinc-finger motifs are underlined. (B) Diagram of the predicted AREB6 protein. Six C2-H2 fingers are represented by filled boxes and one C2-HC finger is represented by a shaded box. Glutamic acid-rich region and Ser/Thr rich region are also indicated.

nucleotides 1761 to 1846 of AREB6 a) by plaque hybridization. The longest clone, AREB6b, and AREB6a were subcloned into pKS and sequenced.

Expression of HEB and AREB6 Protein in Bacteria and DNase I Footprint and Methylation Interference Analyses—AREB1 (nucleotides 1014 to 4126 of HEB) and AREB7 (nucleotides 2055 to 3518 of AREB6) were cut with SalI and NotI in the Superscript vector then filled in with Klenow fragment and subcloned into pGEX-3X vector cut with EcoRI and blunt ended, so that the GST-HEB and GST-AREB6 fusion proteins will be produced. The expressed GST fusion proteins were purified by Glutathione Sepharose column chromatography (Pharmacia). DNase I footprinting was performed as follows. Purified GST-fusion proteins were incubated with ARE probe, 32P-labeled HindIII-EcoRI fragment from pKSARE (MluI-PvuII fragment of Atp1α1 was subcloned into pKS multiple cloning site), in a 15 μl reaction mixture (40 mM HEPES pH 8.4, 60 mM KCl, 0.3 μg of poly d[I-C], 2 μg of BSA) for 20 min at room temperature. Three microliters of 4 μg/ml DNase I in 18 mM MgCl2 and 3 mM CaCl2 was added and the mixture was incubated for 40 s at room temperature. One microliter of 0.1 M EDTA was added to terminate the DNase I digestion. DNA was purified by phenol–chloroform extraction and ethanol precipitation and was resolved by 10% urea-polyacrylamide gel electrophoresis. Methylation interference analysis was performed as follows. Two micrograms of the fusion proteins were incubated with partially methylated ARE probe and resolved by 4% polyacrylamide gel electrophoresis. The free and bound probes were isolated and cleaved with piperidine. DNA was analyzed by 10% urea-polyacrylamide gel electrophoresis (10).

RESULTS

Isolation and Sequencing of cDNA Clones Encoding the Specific ARE Binding Protein—For the purpose of obtaining cDNA clones encoding specific binding factors to the ARE, we screened a HeLa cell expression library with a DNA fragment including the ARE sequence. Four positive clones (AREB1, AREB2, AREB6a, and AREB7) were isolated. AREB1 was identified as a partial clone of HEB

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AREB2 was a nonspecific DNA binding protein. AREB7 was a partial clone of AREB6a. For obtaining AREB6b, the 5'-extended clone of AREB6a, a primer extension library was constructed and screened with a DNA fragment of the 5' region of AREB6a. The combined nucleotide sequence of AREB6a and AREB6b is shown in Fig. 1A. It contains 5,316 nts (excluding polyA tract) and one long open reading frame codes 1,124 amino acids. The putative initiating methionine was located at the 4th nucleotide (numbered as +1). Although the expected mRNA length is around 6 kilobases from Northern analysis (data not shown), we could not get any 5'-extended clone of AREB6b from HeLa and Jurkat cell cDNA libraries. The deduced amino acid sequence revealed that the molecule contains seven putative zinc-finger motifs clustered in groups of four (amino acid residues 172 to 193, 202 to 222, 242 to 262, and 270 to 292) and three (amino acid residues 906 to 926, 934 to 954, and 962 to 981). They are C2-H2 type zinc-fingers (14) except that the fourth finger is a recently identified C2-HC type zinc finger (15). A glutamic acid-rich region is found at the C-terminal portion (52 Glu's out of 134 amino acid residues from 989 to 1123). A Ser/Thr-rich region is observed (24 Ser/Thr out of 79 amino acid residues from 638 to 716) between the two zinc finger clusters (Fig. 1B). Homology search revealed that the obtained clone was apparently an extended clone of Nil-2-a, which was identified as a negative regulator of interleukin 2 gene in Jurkat cells (16). Three nucleotide differences were observed in the coding region between AREB6 and Nil-2-a; 1825G was C in Nil-2-a, 2014G was C and 2042T was C. The nucleotide sequence was totally different after 3492 of AREB6. To exclude the possibility that this is due to a cloning artifact, we performed S1 nuclease mapping using HeLa and Jurkat cell polyA' RNA. The 3'-end labeled DraI (3602)-XbaI (3384) fragment (single-

Fig. 2. DNase I footprinting and methylation interference analyses of the bacterially expressed AREB6 and HEB protein. (A) and (B): DNase I footprint of AREB6 and HEB. Increasing amounts of purified GST-AREB6 fusion protein (A, lanes 2-6 and 8-12) or GST-HEB fusion protein (B, lanes 2-9) were added to the reaction mixture described in "MATERIALS AND METHODS." Protected regions are indicated. Hypersensitive sites are shown by arrows. Maxam-Gilbert G residues are in the adjacent lanes (A, Lanes 1 and 7; B, lanes 1 and 10). (C) and (D): Methylation interference analysis. C denotes bound probes and F denotes free probes. Interfered G residues are shown by closed triangles. Enhanced G residues are shown by open triangles. (E): Summary of DNase I footprinting and methylation interference experiments. The G residues that are interfered with are marked by closed triangles and those that are enhanced are marked by open triangles.

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stranded DNA as a probe) was protected in the presence of HeLa or Jurkat polyA+ RNA, while we did not observe the 107nt fragment which is expected to be detected if there exists an mRNA molecule with a different nucleotide sequence after 3492 from our cDNA clone (data not shown).

Binding of AREB6 and HEB to ARE Sequence—To reveal the binding regions of the AREB6 and HEB proteins, the isolated cDNAs of AREB1 and AREB7 (the clones contained the DNA binding domain of HEB and AREB6, respectively) were subcloned into pGEX-3X vector to express GST-fusion protein. The fusion proteins were purified on a Glutathione Sepharose column. DNase I footprinting analysis indicated that the residues from -100 to -87 of the upper strand (Fig. 2A, lanes 2-6) and -87 to -96 of the lower strand (Fig. 2A, lanes 8-12) were protected from digestion by GST-HEB fusion protein and that the residues from -77 to -68 of the upper strand (Fig. 2B, lanes 2-5) and from -65 to -77 of the lower strand (Fig. 2B, lanes 6-9) were protected from digestion by GST-AREB6 fusion protein. Methylation interference analysis also indicated that 8 guanine residues interfered with and 1 guanine enhanced the formation of the complex with GST-HEB fusion protein (Fig. 2C, lanes 3 and 4; 2D, lanes 4 and 5) and 5 guanine residues interfered with and 3 guanines enhanced the formation of the complex with GST-AREB6 fusion protein (Fig. 2C, lanes 1 and 2; 2D, lanes 2 and 3). The DNase I-protected regions and the patterns of the interfering and enhancing G residues (summarized in Fig. 2E) are distinct from those obtained for C1, C2, and C3 complexes observed by gel retardation analysis of MDCK or B103 cell nuclear extract (8). These results strongly suggested that the isolated cDNA clones code for proteins distinct from the factors in C1, C2, and C3 complexes.

Expression of AREB6 mRNA in Various Tissues and

Table I. Effect of HEB and AREB6 on the Atp1α1 gene expression.

<table>
<thead>
<tr>
<th>Reporter gene</th>
<th>HEB (μg)</th>
<th>AREB6 (μg)</th>
<th>Relative luciferase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pA1U-102LF</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>103±31</td>
<td>239±58 97±2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>117±9</td>
<td>206±47 82±1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>124±7</td>
<td>84±29 34±7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>224±7</td>
<td>56±26 78±6</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>357±7</td>
<td>37±43 48±2</td>
</tr>
<tr>
<td>pA1ED+31LF</td>
<td>0</td>
<td>100</td>
<td>ND  ND</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>422±1</td>
<td>ND  ND</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>459±70</td>
<td>ND  ND</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1,020±9</td>
<td>ND  ND</td>
</tr>
</tbody>
</table>

*Nine micrograms of pCDM8 (expression vector) was co-transfected and relative luciferase activity was normalized as 100. ND: Not determined. Average of two to six independent transfections and standard deviation is shown.

Table II. Effect of HEB and AREB6 on linker substitution mutations of Atp1α1 promoter in COS7 and 3Y1 cells.

<table>
<thead>
<tr>
<th>Reporter</th>
<th>Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pA1U-102LF</td>
<td>393±52   39.6±4.6</td>
</tr>
<tr>
<td>pA1L511LF</td>
<td>342±58   34.5±1.9</td>
</tr>
<tr>
<td>pA1L522LF</td>
<td>370±68   19.2±8.4</td>
</tr>
<tr>
<td>pA1L533LF</td>
<td>408±66   25.7±4.7</td>
</tr>
<tr>
<td>pA1U-61LF</td>
<td>189±52   ND</td>
</tr>
</tbody>
</table>

*Activity is the ratio of the luciferase activity in the presence of pCDM8-HEB or pCDM8-AREB6 to that in the presence of pCDM8 (as 100). Effect of HEB was determined in COS7, while that of AREB6 was determined in 3Y1 cells. ND: Not determined. Average values of two independent experiments involving doublet transfections are given.
Cultured Cell Lines—To establish the tissue distribution of the AREB6 mRNA, we performed Northern hybridization analysis of polyA+ RNAs from various human tissues (Fig. 3). mRNAs were abundant in heart and skeletal muscle, moderately abundant in brain, placenta, and lung, but scarcely detectable in liver, kidney, and pancreas. The approximate size of the mRNA was 6.2 kb in every tissue.

Genomic Southern Analysis of AREB6 Gene—For the purpose of assessing the number of the gene encoding the AREB6, we performed genomic Southern analysis. DNA from human placenta was digested with various restriction enzymes and resolved on agarose gel (Fig. 4). Two hybridized fragments were observed in each lane, suggesting that there are at most two gene loci, possibly only one, in the whole human genome.

Regulatory Effect of HEB and AREB6 on the Atp1α1 Promoter Activity—Specific binding of the HEB and AREB6 proteins was demonstrated as described above. To establish whether these transcription factors modulate promoter activity of Atp1α1, co-transfection experiments were performed using three kinds of cell lines (Table I). In MDCK cells, 3 or 6 μg of the AREB6 expression plasmid enhanced the Atp1α1 promoter activity more than 2-fold while 9 μg of the plasmid had no effect. In 3Y1 cells, AREB6 repressed the promoter activity in a dose-dependent manner. In COS7, no effect of AREB6 was observed. HEB expression plasmid enhanced the expression about 5-fold in COS7 cells, while repression of 23% in MDCK cells and moderate repression of 37% in 3Y1 cells were observed. HEB is known to bind to the E-box consensus sequence, CANNTG, which exists at +50 to +55 of Atp1α1 (7). To eliminate the possible effect of HEB through the E-box consensus in Atp1α1, we constructed pA1ED+31LF, which contains the fragment from −102 to +31 of Atp1α1 fused to the luciferase gene, and tested the effect of the HEB expression plasmid in COS7 cells. The enhancing activity was 12-fold, as shown in Table I. To establish the effects of other factors binding to the ARE region, such as C1, C2, and C3 factors, we examined the effects of HEB and AREB6 on three kinds of linker substitution mutations (8); pA1LS1LF (BglII linker substitution from −73 to −64), pA1LS2LF (from −87 to −78), and pA1LS3LF (from −98 to −89). As shown in Table II, the repression of the promoter activity by AREB6 in 3Y1 cells varies depending on the LS mutations. That is, LS1 showed the same activity as pA1-102LF, LS2 was strongly repressed to as low a level as 19%, and the repression was moderate with LS3. The effect of AREB6 expression plasmid did not change in LS1, in spite of the substitution of the binding site of AREB6. On the contrary, the other linker substitution mutations showed variable effects of these proteins, suggesting complex interactions of ARE binding proteins and various interactions among C1, C2, and C3 factors and AREB6. The enhancing effect of HEB in COS7 cells did not change in any of the linker substitution mutations. HEB did not bind to LS3 mutation, but here again the effect of the HEB expression plasmid did not change. The enhancing effect of HEB was reduced to less than 2-fold when we used the deleted ARE site (pA1U-61LF).

DISCUSSION

Na,K-ATPase α1 subunit gene (Atp1α1) is a typical housekeeping gene, which is ubiquitously expressed in various cell types. The cis regulatory element, ARE, was identified as a common positive regulatory element among various kinds of cell lines of different tissue origins. Four binding factors to ARE, C1, C2, C3, and ATF1, were also identified (8). For the purpose of obtaining cDNA clones specifically binding to ARE, we performed Southern blotting and obtained two specific clones. One of the clones, AREB1, was a partial clone of the human helix-loop-helix protein HEB (13). The binding site of AREB1 to ARE of Atp1α1 does not contain a typical E-box consensus sequence. The other clone, AREB6, was apparently an extended clone of Nil-2-a (16). Two clusters of zinc fingers are composed of four fingers (three C1-H2 type and one C2-HC type) and three (C1-H2 type) fingers. These widely separated clustered structures resemble those of MyTI (myb type transcription factor) (16). The repertoire of the ARE binding proteins was suggested to be quite divergent among different cell types and tissues. HEB is expressed in a tissue-specific manner; it is abundant in thymus and ovary, moderately abundant in kidney, lung, and brain, but absent in spleen, stomach, and heart (13). Likewise, AREB6 also showed tissue-specific expression (Fig. 3). Among other binding factors, C3 is also tissue-specific, while C1, C2, and ATF1 are ubiquitous. Taken together, the housekeeping Atp1α1 is regulated through different combinations and interactions of transcription factors which are ubiquitously or tissue-specifically expressed depending on cell types and tissues.

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The result of the co-transfection experiment indicates that the effects of HEB and AREB6 are not apparently binding site-specific. This may suggest that HEB and AREB6 could interact with other possible binding sites in other regions of Atp1α1 or the vector sequence. In the ARE region, multiple factors such as C1 and C2 in all cell types, and C3 in some specific cell types have been shown to bind (8). The interactions of HEB or AREB6 with other binding factors could influence the activity of the proteins. Actually, the effect of AREB6 was variable, when we used linker substitution mutations of LS1, LS2, and LS3, being defective in C1 and C2 binding, in C1, C2, and C3 binding, and in C3 binding, respectively (Table II). Analysis of the molecular interactions of these factors in a defined in vitro system should reveal the mechanism of the positive and negative effects on transcription of Atp1α1 promoter.

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REFERENCES